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GRANT NUMBER: DAMD17-94-J-4510

TITLE: Study of the Met Tyrosine Kinase in the Pathogenesis of Breast Cancer

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CONTRACTING ORGANIZATION:
Massachusetts General Hospital
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REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 23 Sep 94 - 22 Sep 95

4. TITLE AND SUBTITLE
Study of the Met Tyrosine Kinase in the Pathogenesis of Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4510

6. AUTHOR(S)
T. Jake Liang, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Development of breast cancer is a multistep process involving a complex interplay of both inherited and acquired genetic alterations. In this grant, we have proposed to pursue several lines of studies to define the role of the met tyrosine kinase in the development of breast cancer. First we plan to characterize the structural and functional alterations of the met gene and its product(s) in breast cancers. Second we wish to define the molecular basis of altered met expression in the pathogenesis of mammary epithelial neoplasia. Third we plan to assess the biological consequences of altered met expression with respect to the development and oncogenesis of breast tissues in animal model systems. In this progress report, we have evidence that c-met expression is altered in human breast cancer lines and that activated met oncogene can induce breast cancer development in transgenic mouse model.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer/etiology/c-met gene/oncogene/tyrosine kinase/transgenic	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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I. Introduction

Breast cancer is the most common malignancy affecting women in the Western World. Epidemiological studies have defined certain factors that may contribute to the risk of breast cancer, the most important one being a family history of the disease (1, 2). Approximately 5-10% of breast cancers are associated with inherited susceptibility with one or more autosomal dominant traits. Linkage at the estrogen receptor on chromosome 6 has also been reported in breast cancer family with a late-onset mode (3). Families with germ-line p53 mutations (Li-Fraumeni Syndrome) often have multiple breast cancers, representing only about 1% of all familial breast cancer cases (4). Molecular analysis of sporadic breast cancer DNAs has revealed loss of heterozygosity on chromosomes 1p, 1q, 3p, 6q, 7q, 8q, 11p, 13q, 15q, 16q, 17p, 17q, and 18q (5). Gene amplification is also frequently observed in the c-myc, HER2/neu, and int-2/PRAD-1 genes of breast tumors (6-8). Furthermore, a significant association between the level of nm23 expression and aggressive tumor behavior has been demonstrated (9). Recently, a breast cancer susceptibility gene (BRAC1) in chromosome 17q13 has been identified (10). These observations suggest that breast cancer, similar to the oncogenesis of other solid tumors, develops through a multistep process involving various genetic alterations. Identification and characterization of these genetic alterations will not only offer the possibility of early diagnosis but also spawn new treatment modalities targeted specifically at the altered phenotype of malignant cells.

Tyrosine kinases have been by far the most important class of mutated cellular genes during malignant transformation. The met oncogene was identified in a N-methyl-N'-nitro-N-nitrosoguanidine(MNNG)-treated human osteosarcoma cell line HOS, using the NIH 3T3 cell transfection system (11-13). The activation of the met oncogene was shown to occur via a chromosomal rearrangement, presumably as a result of the mutagenic effect of MNNG. The rearrangement generates a chimeric gene, fusing an upstream promoter-containing sequence (tpr) from chromosome 1 in front of the carboxyl terminus of the met protooncogene on chromosome 7. The fusion molecule (MW 65 KD) contains the tyrosine kinase domain of the met protooncogene. The tpr sequence consists of a constitutive promoter and an open reading frame coding for a protein with strong sequence homology to nuclear oncoproteins fos, jun, transcription factor CREB, and members of intermediate filament multigene family (14). The common feature

among these molecules is that they contain a leucine zipper which has been shown to be required for dimerization and activation of these proteins (15). Recent data demonstrated that tpr-met oncogene was indeed activated through this leucine zipper interaction, resulting in a constitutively phosphorylated and presumably active state of this tyrosine kinase molecule (16). Overexpression of normal c-met appeares to be sufficient to activate the tyrosine kinase, which may explain the transforming potential of amplified c-met gene in some human tumors (17). The identification and characterization of other forms of c-met has demonstrated that abnormal processing of the extracellular domain of the protein can also result in constitutive activation of c-met (17). Similar to what has been described for other receptor tyrosine kinases (RTK), such as trk and ret proto-oncogenes, mutations affecting the extracellular or transmembrane domain may be the molecular basis for the oncogenic potential of met in some human cancers (18-21).

The proto-oncogene of met encodes a receptor of 190 kDa protein, conposed of two disulfide-linked subunits: an extracellular 50 kDa α-subunit and a transmembrane 145 kDa β-subunit. The receptor is synthesized as a 170 kDa precursor that is glycosylated and cleaved posttranslationally to give the mature heterodimer. The intracellular domain of c-met protein has a structure resembling that of the protein tyrosine kinase (PTK) family. Recent studies have shown the presence of multiple forms of c-met gene products, presumably as a result of alternative splicing (22). The functional significance of these alternative forms has remained largely unknown.

Recently, hepatocyte growth factor (HGF) has been shown to be the ligand for the c-met receptor (23). HGF is also known as hepatopoietin and is identical to scatter factor, which affects the motility, chemotaxis, and invasiveness of epithelial and endothelial cells in culture (24-26). HGF has been shown to be the most potent growth factor for rat and human hepatocytes in primary cultures (27). In addition to its mitogenic effect, it regulates cellular shape as a morphogen and cellular motility as a motogen (24). The pleoitropic effects of HGF-SF on cells suggest a complex interplay of receptor-mediated signal transductions. It is clear from previous studies that HGF appears to induce rather diverse biological effects in various cell types (24). The factors determining the outcome of HGF actions have remained largely unknown. Elucidation of these factors will undoubtedly give us some unique insights into the

molecular mechanisms of growth regulation and malignant transformation.

Cytogenetic studies of transformed cells derived from human malignancies suggested that the mechanism of chromosomal rearrangement resulting in fusion gene products with transforming potential may be a common mechanism of oncogenesis (25). Several examples are the Philadelphia chromosome of chronic myelogenous leukemia, t(14;18) translocation of follicular B cell lymphoma, and t(10;14) translocation of T cell acute lymphoblastic leukemia. Therefore, activation of met proto-oncogene via this mechanism may be more than an in vitro observation in the original mutagenesis experiment described above. In addition, c-met proto-oncogene has been shown to be amplified and overexpressed in a human gastric tumor cell line, although the mechanism of activation is distinct from that of tpr-met activation (26). A recent report described the finding of similar chromosomal rearrangements resulting in tpr-met fusion gene in human gastric tumors (27). This provocative finding is consistent with the observation that nitroso compounds are epidemiologically associated with the occurrence of gastric cancer in humans (28).

Regardless of the oncogenic potential of the activated met protein, its proto-oncogene product likely plays an important role in the growth and differentiation of epithelial cells in various organs. Recent studies have demonstrated a wide tissue distribution of this protein, such as in breast, intestine, stomach, liver, pancreas, kidney, etc., and more interestingly, increased level of met RNA and protein in several carcinoma specimens, particularly in thyroid, gastric and intestinal tumors (29). In contrast, none of 15 primary breast cancers showed expression of met protein, whereas significant met expression was detected in 4 of 4 normal mammary epithelium (29). Another provocative study demonstrated that the c-met locus on chromosome 7 (7q21-22) was deleted in 41% of 245 patients with primary breast cancer (30). In addition, patients with loss of heterozygosity on chromosome 7q21-22 had significantly shorter metastasis-free survival and overall survival. Recent studies on the functions of HGF-met activation have suggested a potential regulatory role for c-met in the morphogenesis of breast tissues (31). These observations, in toto, suggest that this region of chromosome 7, possibly the c-met gene, may be the site of a breast tumor or metastasis suppressor gene. The concept of a RTK as a tumor suppressor gene has been substantiated by a recent report demonstrating that the RET tyrosine kinase gene appears to

code for the tumor susceptibility gene in Multiple Endocrine Neoplasia Type II Syndrome (MEN II) (32).

In this grant application, we plan to search for genetic abnormalities affecting the c-met gene at the DNA, RNA, and protein levels in human breast cancer cell lines and tumor specimens (Specific Aim A), and study the key cellular targets of c-met gene product in mammary epithelial cells and define the functional effects of mutated met proteins through exploring a innovative technology to identify and characterize interacting proteins (Specific Aim B). We are applying the two-hybrid system in yeast in order to clone cellular genes whose protein products interact specifically with the met protein. As Specific Aim C, we plan to develop animals models to examine the oncogenic potentials of met oncogene and to assess the role of met proto-oncogene in the growth and development of breast tissues.

II. Body

We are applying a variety of molecular and cellular approaches to study the role of c-met in the development of breast cancer. We have generated significant amounts of preliminary data regarding each of the specific aims. For Specific Aim A, we have examined the status of c-met gene and its product(s) in several breast cancer cell lines and have observed that the expression of met gene in these lines appeared to be absent or altered with aberrant forms of gene product (Table 1). For Specific Aim B, we have initiated our effort to clone cellular target(s) of c-met in order to understand the signal transduction pathway leading to malignant transformation of mammary epithelium. We have identified several candidate genes whose gene products interact specifically with an intracellular domain of c-met in the yeast two-hybrid system. Further characterization of these clones, hopefully, will lead to a better understanding of how mutated c-met induces breast cancers. Finally for Specific Aim C, we have generated four transgenic founder lines expressing the met oncogene (tpr-met). In line one, 3 of 8 developed breast cancers by 12 months of age (Table 2). The other two lines, which are less than 6 months of age, have developed lymphoma and proliferative abnormalities in their stomachs and livers. In addition to continuing our analyses of these lines, we hope to extend our efforts to study the biological effects of inactivated met gene in transgenic mice with germ-line disruption of the c-met gene. By exploring these three distinct but complementary approaches, we

hope to gain important insights into the multistep pathogenesis of breast cancer.

III. Conclusions

From our preliminary studies on genetic alterations involving the c-met gene, we observed a significant association with tumorigenicity of cell lines and aberrant expression of c-met (Specific Aim A). This observation is consistent with our hypothesis that dysregulated synthesis of c-met plays a role in breast carcinogenesis.

We have performed extensive studies in the transgenic model of met (Specific Aim C). The pattern and occurrence of mammary hyperplasia and tumors in the tpr-met transgenic strains support strongly the conclusion that these lesions are a direct effect of the transgene. Hyperplasia and tumors developed in all three independent transgenic lines (MTM1, MTM2, and MTM3). In addition, no spontaneous mammary tumors developed in any of the nontransgenic littermates maintained in parallel. This observation is consistent with other investigator's experiences with the FVB strain. Furthermore, occurrence of multiple independent mammary tumors, as observed in some of our animals, is rare in spontaneous breast tumors of normal mice. The use of a cellular promoter (metallothionein) rather than the MMTV promoter to drive the tpr-met transgene further speaks for the uniqueness of this animal model in the study of mammary carcinogenesis. It is interesting to note that only breast tumors expressed high-level of the tpr-met transcript and protein, whereas normal tissues including mammary epithelium expressed very little. Perhaps, during physiological hyperplasia of mammary glands as a result of pregnancy, cells expressing higher levels of the oncoprotein were selected and clonally expanded to eventually form foci of mammary malignancy. This observation suggests that mammary adenocarcinoma developed in this transgenic model as a direct effect of high-level expression of tpr-met.

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V. Appendix

Table 1: Altered c-met Expression in Breast Cell Lines.

Cell Line*	Tumorigenic**	DNA***	RNA	Protein
_75N	-	+	+	
70N	-	+	+	+
81NN	-	+	+	
21NT	-	+	+	+
21PT	-	+	+	
18-2-1	-	+	+	
1436N1	-	+	+	
HBL-100	-	+	+	+
Hs578Bst	-	+	+	+
MDA-MB-157	+	+	-	
MDA-MB-231	+	+	-	
MDA-MB-361	+	+	-	
MDA-MB-435	+	+	+	+
MDA-MB-436	+	+	+	
MDA-MB-468	+	+	-	
BT-474	+	+	-	
BT-549	+	+	-	-
MCF7	+	+	-	-
MCF10A	+	+	+	
T-47D	+	+	-	
ZR-75-1	+	+	-	-
ZR-75-3	+	+	-	-
DU4475	+	+	-	-
Hs578T	+	+	+	-
SK-BR3	+	+	+	-

* The primary mammary cells 76N and 70N, and immortalized breast cell lines 21-NT, 21-PT, 18-2-1, and 1436N1 are from Ruth Sager; the remaining cell lines are obtained thru ATCC.

* * "Nontumorigenic" is defined as inability to form tumor in nude mouse.

* * * Absence of gross gene rearrangement by restriction and Southern blot analysis is defined as "+".

Table 2: Tumor Incidence in MTM Transgenic Strains

Transgenic Line	Mice in continuous breeding	Mice with mammary tumors*	Idependent mammary tumors	Mean Age of Onset#	Mice with other tumors@
MTM1*	10	5	6	317	3
MTM2	8	2	5	394	1
MTM3	8	2	3	433	2

Cohorts of mice in each of the MTM transgenic strains were observed for tumor development. Mammary and other tumors were observed in all strains.

* Parallel cohort of nontransgenic lttermates (16) were set up for continuous mating as controls and none of the mice developed tumors at two years of age.

\# Data reported as mean age in days of detection of breast tumors.

@ These animals were not included in the cohort of female mice in continuous breeding. One female MTM1 animal developed diffuse lymphoma at 9 months of age and a male had metastatic spindle cell sarcoma at two years of age. An orbital osteosarcoma was noted in the third male MTM1 animal at 18 months of age. One MTM2 male mouse was noted to have two lesions: one squamous papilloma and the other rectal spindle cell sarcoma. Two female MTM3 animals developed lymphoma, one thymic and the other diffuse at an early age of three months.